IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: OKAMURA=2B

In re Application of:	)	Group Art Unit: 1646
	)	
H. OKAMURA et al.	)	Examiner: Dong Jiang
	)	
Appln. No.: 09/050,249	)	Washington, D.C.
	)	
Date Filed: March 30, 1998	)	Confirmation No. 6601
	)	
For: IFN-GAMMA PRODUCTION INDUCING PROTEIN...	)	

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner for Patents
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Sir:

1. I, Tsunetaka Ohta, declare as follows:

2. I am a citizen of Japan, residing at 3-1015-48 Hirai, Naka-ku, Okayama 703-8282, Japan.

3. In 1979, I received a bachelor of B. S. in Pharmaceutical Science from Hiroshima University, Hiroshima, Japan; and in 1985, I received a doctorate of Ph. D. in Biochemistry of Cell Cycle from the above-identified Hiroshima University.

4. As shown in my curriculum vitae attached hereto as Exhibit A, from 1985 to 2011, I have researched and directed in Hayashibara Biochemical Laboratories, Inc., fundamental studies and industrial applications of physiologically active substances, particularly, interferons and interleukins, specifically interferon-γ inducing factor (IGIF) or interleukin-18 (IL-18). While, from 2002 to 2011, I have been in a position as a visiting Professor (Head of collaborative laboratories) in Applied Technology for Molecular and Cellular Biology, Graduate School of Biosphere Science, Hiroshima University.

5. I have read and am thoroughly understood the present invention (US 09/050,249) and the contents of Nakamura et al. , *Infection and Immunity.*, Vol. 61, No. 1, pp. 64-70, 1993; Ailsa M. Campbell, *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 13, Chapter 1, pp. 1-33, 1984; Marc Shulman et al., *Nature*, Vol. 276, pp. 269-270, 1978); and United States Patent No. 5,358,850, cited by an examiner in official actions in the procedure of the present invention.

6. I hereby declare that any researchers at the time the present invention was made neither have been motivated to try to obtain a monoclonal antibody which binds to a factor which provides a costimulatory signal for gamma interferon production (called "Nakamura's Factor", hereinafter), disclosed in the above-identified Nakamura's publication (abbreviated as "Nakamura", hereinafter); nor have succeed in obtaining such a monoclonal antibody based on the above-identified publications, particularly, Nakamura, for the reasons as stated below:

Reason 1: Possibility of motivation for trying to obtain any monoclonal antibody which binds to "Nakamura's Factor"

It is reported in "Abstracts" of Nakamura that:

> "*A protein factor which induces high levels of gamma interferon (IFN-γ) in resting splenic nonadherent cells was isolated from the sera of mice with generalized inflammation caused by endotoxin shock.*"

However, any researchers would never make themselves understood that Nakamura's Factor was worth a substance suitable for preparing a monoclonal antibody which binds to Nakamura's Factor and nobody has been motivated to try to obtain any such monoclonal antibody for the following reasons:

Firstly, Nakamura purified Nakamura's Factor on SDS-PAGE and obtained a purified specimen (50 to 55 kDa), confirmed by staining with silver. As well known, the staining with silver employed in Nakamura (see, FIG. 2) results in staining of nucleic acids and saccharides, as well as proteins. Accordingly, the single band (50 to 55 kDa) on SDS-PAGE (lane c) in FIG. 2 may be a nucleic acid or saccharide or something else. This speculation would certainly be supported by:

> "*Since the factor lost its activity in SDS-PAGE, we also failed to definitely establish that the band revealed by SDS-PAGE was the factor.*" (see, page 68, right column in Nakamura).

Secondary, from my knowledge of mouse IGIF or IL-18, the specific activity of 283,333 U/mg protein, which had been purified from a crude serum specimen with a specific activity of 34 U/mg protein, through only the four-purification steps of "Ammonium sulfate", "DEAE-Sepharose", "Ultrogel", and "Phenyl-Sepharose" as shown in "TABLE 1", page 66, of Nakamura, was too high in purification level, when regarding

the finally purified specimen as mouse IGIF or IL-18 prepared from the crude serum specimen. I presume that such a high specific activity (283,333 U/mg protein) is due to mouse natural killer stimulatory factor (NKSF/IL-12) alone or presumably in combination with the targeted Nakamura's Factor or something else.

Thirdly, since at the time Nakamura reported their finding, NKSF/IL-12 had been cloned already and therefore they could have examined whether Nakamura's Factor was a novel factor different from other known substances, particularly, NKSF/IL-12.

However, they didn't and merely stated that:

> "*It will be interesting to examine its relationship to NKSF/IL-12 and to speculate on its roles relevant to the regulation of the Th1 subset, which is said to be involved in cellular immunity in vivo.*", in the last paragraph, page 69, of Nakamura.

This means that the reality of Nakamura's Factor has not yet been revealed or elucidated.

Under these facts or circumstances, the technical disclosure of Nakamura would never have motivated any researchers to try to prepare any monoclonal antibody which binds to Nakamura's Factor.

Reason 2: Possibility of success of obtaining a monoclonal antibody which binds to mouse IGIF or IL-18 based on Nakamura

According to "Purification of the factor" in "RESULTS", pp. 65 to 66, of Nakamura, Nakamura's Factor was prepared from 300 ml sera collected from mice, which had been treated with *Propionibacterium acnes* and LPS. Since the volume of serum available from an adult mouse is generally about one milliliter, it can be estimated that Nakamura subjected about 300 (300/1=300) mice to their experiment.

Figure 2 A in Takashi Nishioka et al., *Journal of Leukocyte Biology*, Vol. 82, pp. 327-334, 2007, as attached as "Reference 1", shows that the serum IL-18 level in a mouse, treated with *Propionibacterium acnes* and LPS similarly as in Nakamura, is about 5 ng/ml. Therefore, even if the above-identified pooled mouse serum (300 ml) in Nakamura contained mouse IGIF or IL-18, it can be calculated to contain about 1.5 μg [(about 5 ng/ml) x 300 ml ≒ 1.5 μg)] of mouse IGIF or IL-18 in total. Actually, since such mouse IGIF or IL-18 in the serum must be purified for use as an antigen, the amount of the above-identified mouse IGIF or IL-18 is inevitably lowered after prescribed purification steps. In this regard, even if Nakamura's Factor contained mouse IGIF or IL-18, it was only yielded in an amount of 0.26 μg from "Phenyl-Sepharose" in a recovery of 17%, as shown in Table 1 of Nakamura.

While, Example 3-3, captioned with "*Preparation of hybridoma M1*" in the present specification, the present inventors immunized a rat with mouse IGIF or IL-18 four times at respective doses of 20 μg/rat, summing up to 80 [(20 μg) x 4 = 80 μg)] μg in total.

According to the above Example 3-3, even used for immunizing only one rat, at least about 16,000 (80/1.5 x 300 heads = 16,000) mice is required to prepare a requisite amount of antigen for preparing a desired hybridoma, provided that the above-identified pooled mouse serum (300 ml) contained mouse IGIF or IL-18 in an amount of about 1.5 μg and recovered in a 100% yield after purification steps. While, considering the above-mentioned actual yield after purification steps, at least about 92,000 (80/0.26 x 300 heads ≒ 92,308) mice is required to prepare a desired hybridoma.

Experiment using such a large number of mice is beyond an actually-practicable laboratory scale due to its heavy labors and costs, and it must certainly be avoided from recent zoophilism.

Under these facts or circumstances, possibility of success of obtaining a monoclonal antibody, which binds to mouse IGIF or IL-18, by using mice sera as indicated by Nakamura would be definitely denied.

7. Conclusion:

As evident from the above, I declare that any researchers at the time the present invention was made would have never been motivated to try to prepare any monoclonal antibody which binds to Nakamura's Factor even if, in fact, it contains or does not contain mouse IGIF or IL-18. Further, I also declare that it is substantially difficult to obtain any monoclonal antibody which binds to IGIF or IL-18 by using Nakamura's Factor as an antigen for the reasons as stated above.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Tsunetaka Ohta

NAME: Tsunetaka Ohta

Sep. 1, 2011

DATE: 1st day of September, 2011

Attachment:

Exhibit A: Curriculum vitae of Tsunetaka Ohta
Reference 1: Takashi Nishioka et al., *Journal of Leukocyte Biology*, Vol. 82, pp. 327-334, 2007

CURRICULUM VITAE

Sep. 1, 2011

NAME:	Tsunetaka Ohta
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PRESENT ADDRESS:	3-1015-48 Hirai, Naka-ku, Okayama 703-8282, Japan Tel: +81-86-274-3247, Fax: +81-86-274-3247
MARITAL STATUS AND FAMILY:	Married, No children
PRESENT POSITION:	Director Biomedical Institute, Research Center, Hayashibara Biochemical Laboratories Inc. & Visiting Professor Applied Technology for Molecular and Cellular Biology (collaborative lab.), Graduate School of Biosphere Science, Hiroshima University 675-1 Fujisaki, Naka-ku, Okayama 702-8006, Japan Tel: +81-86-276-3141, Fax: +81-86-276-6885 e-mail: t_ohta@hayashibara.co.jp, tohta@hiroshima-u.ac.jp

DEGREES, LICENSES, AND CERTIFICATES:

B. S. in Pharmaceutical Science	1979	No.5002009 Hiroshima University, Hiroshima, Japan
Pharmacist's License	1979	No.179644 National Pharmacist's Board of Japan
M. S. in Biochemistry	1982	No.4956 Hiroshima University, Hiroshima, Japan
Ph. D. in Biochemistry of Cell Cycle	1985	No.527 Hiroshima University, Hiroshima, Japan
Narcotic Drugs Researcher License	2003	No. 160001 Okayama Prefecture, Okayama, Japan
Health Administer License	2004	No.33000446981 Okayama Labor Bureau, Okayama, Japan
Certificate of Bioinformatician	2007	No.7-113 Japanese Society for Bioinformatics

RESEARCH AND EMPLOYMENT EXPERIENCES:

1985 - 1987	Fujisaki Institute, Hayashibara Biochemical Labs., Inc. 675-1 Fujisaki, Okayama 702, Japan	Research Associate
1987 - 1988	Fujisaki Institute, Hayashibara Biochemical Labs., Inc.	Senior Scientist & Project Leader
1988 - 1990	G. I. Oncology The MGH Cancer Center, Harvard Medical School Bldg. 149, 13th Street, Charlestown, MA 02129, USA	Research Fellow
1991 - 1995	Fujisaki Institute, Hayashibara Biochemical Labs., Inc.	Senior Scientist & Assistant Director
1995 - 2004	Fujisaki Institute, Hayashibara Biochemical Labs., Inc.	Chief Scientist & Sub-Director
1998 - 1999	Graduate School of Okayama Medical School 2-5-1 Shikata-cho, Okayama 700-8558, Japan	Lecturer
2002 - present	Applied Technology for Molecular and Cellular Biology, Graduate School of Biosphere Science, Hiroshima University 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528, Japan	Visiting Professor (Head of collaborative lab.)
2004 - 2005	HSP Research Institute, Inc. 2-8 Doshomachi, 2 Chome, Chuo-ku, Osaka 541-8510, Japan	General Manager
2004 – 2006	Fujisaki Institute, Hayashibara Biochemical Labs., Inc.	Director
2006 – present	Biomedical Institute, Research Center, Hayashibara Biochemical Labs., Inc.	Director
2008 – present	Kibi Pharmaceutical Plant, Hayashibara Biochemical Labs., Inc., 416-3 Yoshikawa, Kibichuo-cho, Kaga-gun, Okayama 716-1241	Manufacturing Supervisor for Medicinal Chemicals

SOCIAL ACTIVITIES:

2004 – 2011	Committee for Regulation and Standard, Japan Health Science Foundation	Member
2005 – present	Research Group for Biomarkers in the Next Generation, Japanese Society of Bioinformatics	Organizer
2007 – 2008	Committee for Potential Assessment of Bio-industrial Innovation by Animals and Plants Factories, Chugoku Industrial Innovation Center	Member
2009 – 2011	Medical Techno OKAYAMA, The Okayama Medical Foundation	Vice-president

2009 – 2011 Society of Okayama Medical Technology Vice-president

ACADEMIC SOCIETIES:

The Japanese Biochemical Society
Japanese Cancer Association
American Association for Cancer Research
Japanese Society for Immunology
The Molecular Biology Society of Japan
The Japanese Society for Wound Healing
International Technology Exchange Society
Japanese Society for Bioinformatics
Japanese Society for Drug Delivery System
International Society to Advance Alzheimer Research and Treatment

AWARDS:

"Highlighted paper selected by Editor-in chief"
T. Tatefuji, C. Arai, T. Mori, Y. Okuda, T. Kayano, A. Mizote, T. Okura, M. Takeuchi, **T. Ohta**, and M. Kurimoto (2006) "The effect of AgK114 on wound healing" Biol. Pharm. Bull. **29**, 896-902.

"ITE Yeager-Kozawa Award"
T. Ohta and S. Fukuda (2007) "Hibernation study with Syrian hamsters" ITE Lett. Batt. New Technol. Med. **8**, 504-508.

"Highlighted paper selected by Editor-in chief"
S. Koya-Miyata, H. Ohta, K. Akita, S. arai, **T. Ohta**, T. Kawata, and S. Fukuda (2010) "Cyanine dyes attenuate cerebral ischemia and reperfusion injury in rats." Biol. Pharm. Bull. **33**, 1872-1877.

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<PUBLICATIONS>

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Induction of serum IL-18 with *Propionibacterium acnes* and lipopolysaccharide in phagocytic macrophage-inactivated mice

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Abstract: IL-18, an important regulator of immune responses, is expressed in activated macrophages and also in nonimmune cells, such as keratinocytes and epithelial cells. Increased levels of serum IL-18 are reported in patients with a wide variety of diseases, but it is unclear which type of cell is the major source of serum IL-18. Here, we showed that the administration of liposomes encapsulating clodronate (Clo-lip) in mice selectively depleted F4/80$^+$ phagocytic macrophages in the liver and spleen. Serum levels of mature IL-18 with 18 kDa were increased markedly in mice treated with *Propionibacterium acnes* and LPS, whereas administration of Clo-lip and gadolinium chloride, another widely used macrophage inactivator, showed no obvious effect on serum IL-18 levels, which were marginal in the liver, lung, and spleen and more pronounced in the intestines, especially in the duodenum. Treatment with *P. acnes* alone induced IL-18 more than twofold in each organ, and *P. acnes* and LPS induced a marked increase in IL-18 levels in the liver and spleen but decreased in the intestines. The administration of Clo-lip showed only a marginal effect on the IL-18 levels in these organs. Furthermore, serum levels of liver enzymes and TNF-α and liver injury (necrotic change and granuloma formation) induced by *P. acnes* and LPS were reduced moderately by Clo-lip. These results suggest that phagocytic macrophages do not actively contribute to the induction of serum IL-18 and liver injury in mice treated with *P. acnes* and LPS. *J. Leukoc. Biol.* 82: 327–334; 2007.

Key Words: cytokine · inflammation · macrophages · mucosal cells

INTRODUCTION

IL-18 was identified originally as an IFN-γ-inducing factor from a murine liver cell cDNA library generated from mice primed with heat-killed *Propionibacterium acnes* and subsequently challenged with LPS [1]. IL-18 is produced intracellularly as an inactive, 24-kDa precursor form (proIL-18) and secreted as an 18-kDa mature form after cleavage by caspase-1, originally designated IL-1β-converting enzyme [2–4]. IL-18 is now recognized as a multifunctional regulator of innate and acquired immune responses through its activation of Th1 and Th2 responses [2–5]. IL-18 has also been suggested to be a potent, proinflammatory cytokine, which regulates autoimmune and inflammatory diseases [2–4].

Recent studies showed that IL-18 is identified, not only in activated macrophages, including dendritic cells (DC) and Kupffer cells, but also in nonimmune cells, such as keratinocytes, osteoblasts, adrenal cortex cells, epithelial cells of various organs and tissues, microglial cells, and synovial fibroblasts [2–4]. This wide range of distribution implies that IL-18 plays physiological roles and acts as a component of immune regulation.

Increased levels of IL-18 have been reported in the sera from patients with a wide variety of diseases, including autoimmune and inflammatory disorders [6–11], allergy [12], allograft rejection [13], and infectious diseases [14–16], and the elevated serum IL-18 levels are considered to be a parameter for the disease severity and a diagnostic marker. We have shown recently that human oral epithelial cells constitutively express a precursor form of IL-18, stimulation of the cells with neutrophil proteinase 3 (PR3) and LPS induces the secretion of an active form of IL-18 after IFN-γ priming [17], and PR3 activates the cells through a G protein-coupled protease-activated receptor 2 (PAR2) on the cell surface in vitro [18, 19]. Subsequently, we also revealed that neutrophil recruitment and PAR2 activation are critically involved in the induction of serum IL-18 in mice treated with heat-killed *P. acnes* and LPS in vivo [20].

It is still unclear whether the major source of serum IL-18 is activated macrophages or nonimmune cells, such as those of epithelial origin in vivo. A macrophage "suicide" technique, using liposomes encapsulating dichloromethylene bisphosphonate (clodronate), specifically depletes phagocytic macrophages but not neutophils and DC within 1 day or 2 of the i.v. injection of such liposomes into mice or rats [21–23]. It is also reported that i.v. injection of gadolinium chloride ($GdCl_3$) not only blocks phagocytosis of Kupffer cells but also eliminates

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Received October 2, 2006; revised April 10, 2007; accepted April 25, 2007. doi: 10.1189/jlb.1006598

these cells [24, 25]. These techniques have allowed us to investigate whether the major source of serum IL-18 is activated macrophages in mice treated with *P. acnes* and LPS. We also examined the effect of macrophage inactivation by the liposomes on liver injury in the mice.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (6–9 weeks old), obtained from the Institute for Experimental Animals of the Tohoku University Graduate School of Medicine (Sendai, Japan), were used for the experiments.

Bacteria and reagents

P. acnes was grown in brain-heart infusion medium (Difco Laboratories, Detroit, MI, USA) with L-cysteine and Tween-80, as described previously [20]. The harvested bacteria were washed with sterile, distilled water, killed by heating at 60°C for 1 h, and then lyophilized. The lyophilized bacteria were next suspended in PBS (5 mg/ml) and used to prime the mice. LPS from *Escherichia coli* O55:B5 and clodronate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse recombinant (mr)IL-18 was obtained from Medical and Biological Laboratories (Okayama, Japan). Rabbit anti-mouse IL-18 polyclonal antibody (pAb) was provided by Tomaki Hoshino (Kurume University, Kurume, Japan). All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Treatment of mice with clodronate-liposomes (Clo-lip) or $GdCl_3$

A suspension of liposomes encapsulating clodronate was prepared according to a method described previously [22, 23]. Briefly, 75 mg phosphatidylcholine and 11 mg cholesterol were dissolved in chloroform (20 ml) in a round-bottomed flask (1000 ml). The thin film, which formed on the walls of the flask after rotary evaporation at 37°C, was dispersed by gentle shaking for 10 min in 10 ml clodronate solution (200 mg/ml) in PBS. This suspension was kept for 2 h at room temperature, then sonicated for 3 min (50 Hz), and kept for another 2 h. The resulting liposomes floating on the aqueous phase were collected using a Pasteur pipette, suspended in 10 ml PBS, and centrifuged at 5000 *g* for 30 min. The precipituted liposomes were finally suspended in 4 ml PBS; this preparation is referred to as the "original" suspension of Clo-lip. This original suspension was diluted (as described in the text) using PBS, and the diluted suspension was injected i.v. at 0.2 ml/mouse.

The mice were also injected i.v. with $GdCl_3$ (10 mg/kg) or saline [25].

Histological analysis

Immunohistochemistry was conducted as follows. Tissues were fixed in periodate-lysine-4% paraformaldehyde for 6 h at 4°C. After washing in PBS containing sucrose, fixed tissues were embedded in OCT compound (Sakura, Tokyo, Japan) and frozen immediately. Frozen tissue sections (6 μm) were incubated with rat anti-mouse F4/80 mAb (Serotec, Oxford, UK) overnight at 4°C. After that, the sections were treated with peroxidase-blocking reagent (Dako Cytomation, Tokyo, Japan) for 20 min and secondary antibodies, such as the goat anti-rat simple-stain mouse MAX-PO (Nichirei, Tokyo, Japan). The chromogen used was 3',3-diaminobenzidine tetrahydrochloride (Dako Cytomation). The sections were counterstained with hematoxylin. As a negative control, rat isotype-matched control Ig G2b (BD Biosciences, San Diego, CA, USA) was used.

For histopathological analysis, formalin-fixed samples were embedded in paraffin and stained with H&E.

Treatment of mice and preparation of serum and tissue extracts

The mice were injected i.p. with heat-killed *P. acnes* (1 mg dry weight/mouse), and 7 days later, they were challenged i.v. with LPS (1 μg/mouse). As *P. acnes*-primed mice started to die of endotoxin shock 4 h after the LPS challenge [20], blood and tissues were taken from the mice 2 h after the LPS challenge in this study. Blood was collected directly into test tubes following their decapitation, and the serum was recovered by centrifugation at 2000 *g* at 4°C, after which it was stored at −80°C until use. Frozen tissues or organs were homogenized in RPMI 1640 containing Triton X-100 (5 μl/ml), HEPES (10 μmol/ml), BSA (100 μg/ml), gentamicin sulfate (50 μg/ml), and proteinase inhibitor cocktail (10 μl/ml) [26]. The cocktail contains 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64. The supernatants obtained by centrifugation at 10,000 *g* for 10 min at 4°C of the homogenates were then stored at −80°C until use. The experimental procedure followed in this study was approved by the Ethical Board for Non-Human Species of the Tohoku University Graduate School of Medicine (Sendai, Japan).

Measurement of cytokines and liver enzymes

The levels of IL-18 and TNF-α in the samples were determined using a mouse IL-18 ELISA kit (Medical and Biological Laboratories, Woburn, MA, USA) and a mouse TNF-α OptEIA ELISA kit (BD PharMingen, San Diego, CA, USA), respectively. According to the manufacturer, the IL-18 ELISA kit mainly detects an 18-kDa, mature form, and the sensitivity to the precursor form was less than 10% compared with the mature form. The amount of IL-18 in each tissue was expressed as μg/g wet tissue.

Serum asparate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured photometrically using commercial kits (Wako Pure Chemical Industries, Osaka, Japan).

Western blotting

All samples were solubilized with Laemmli sample buffer [27]. SDS-PAGE was performed in a 15% polyacrylamide slab gel under reducing conditions, according to the method of Laemmli [27]. Proteins were transferred to a polyvinylidene difluoride membrane using a semidry transblot system (Atto Instruments, Tokyo, Japan). The blot was blocked for 2 h with 3% w/v nonfat dry milk and 0.05% Tween 20 in PBS (Blotto/Tween) and incubated with anti-mouse IL-18 pAb at 6 μg/ml in Blotto/Tween overnight at 4°C. The blot was washed four times with Blotto/Tween and then incubated for 90 min with HRP-conjugated, affinity-purified goat anti-rabbit IgG at 1:3000 (Pierce Biotechnology, Rockford, IL, USA) in Blotto/Tween. After being washed, IL-18 was visualized with West Femto maximum sensitivity substrate (Pierce Biotechnology). The molecular weight of the proteins was estimated by comparison with the position of a standard (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Experimental values were expressed as the mean ± SD, and the statistical significance of differences between two means was evaluated by one-way ANOVA using the Bonferroni or Dunnett method, for which values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Effect of macrophage inactivation on the induction of serum IL-18 in mice

As it is suggested that activated macrophages, such as Kupffer cells, are the major source of serum IL-18 in vivo [1, 2], we examined the effect of macrophage inactivation by Clo-lip or $GdCl_3$ on the induction of serum IL-18 in mice after treatment with *P. acnes* and LPS. Clo-lip was administered in mice on Days 0, 3, and 5, and the livers and spleens were taken on Day 8. $GdCl_3$ was administered in mice on Days 0, 4, and 7, and the livers and spleens were taken on Day 9. The results from immunohistochemistry showed that the administration of Clo-lip eliminated $F4/80^+$ macrophages (Kupffer cells) in the liver (**Fig. 1A**). $F4/80^+$ macrophages were abundant in the red pulp of the spleen, and the Clo-lip treatment also eliminated $F4/80^+$ macrophages in the spleen (Fig. 1B). In contrast, $GdCl_3$ did not deplete $F4/80^+$ macrophages in the liver and spleen. These

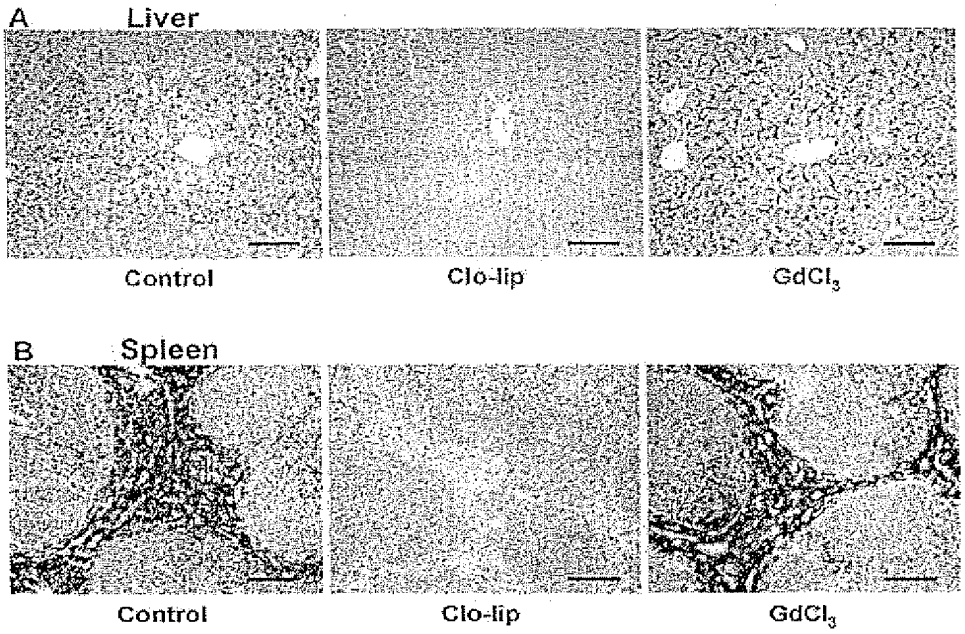

Fig. 1. Effect of Clo-lip and $GdCl_3$ on the depletion of macrophages in mice. Saline (Control) or Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administered i.v. in C57BL/6 mice on Days 0, 3, and 5, and the livers and spleens were taken on Day 8. $GdCl_3$ (10 mg/kg) was administered i.v. in C57BL/6 mice on Days 0, 4, and 7, and the livers and spleen were taken on Day 9. The liver (A) and spleen (B) were subjected to immunostaining with F4/80 antibody and visualized using diaminobenzidine (brown). The results are representative of three mice. Original scale bars, 200 μm.

results indicate that Clo-lip efficiently eliminates phagocytic macrophages in mice; however, it remains possible that $GdCl_3$ inhibits the functions of macrophages, even though it did not eliminate them [24]. Therefore, we used Clo-lip and $GdCl_3$ in the next experiments.

Serum IL-18 levels were increased markedly by treatment with *P. acnes* and LPS, whereas the administration of Clo-lip, three times (1 day before and 2 and 4 days after *P. acnes* injection), or $GdCl_3$, three times (2 days before and 3 and 5 days after *P. acnes* injection), showed no obvious effect on the serum IL-18 levels (**Fig. 2A**). Consistent with this, Western blot analysis showed that an 18-kDa, mature form of IL-18 was detected in the sera of *P. acnes* and LPS-treated mice and that the band was unchanged by Clo-lip or $GdCl_3$ treatment (Fig. 2B). These results indicate that activated macrophages are not critically involved in the induction of serum IL-18 in mice treated with *P. acnes* and LPS and suggest that other cells are the source of serum IL-18.

As Clo-lip and $GdCl_3$ showed the same effect on the induction of serum IL-18 levels, and Clo-lip efficiently eliminated $F4/80^+$ macrophages in mice, we used Clo-lip in the following experiments.

IL-18 levels in various organs in mice

We then examined the IL-18 levels in various organs in mice. In untreated, control mice, the IL-18 levels were marginal in the liver, lung, and spleen and more pronounced in the intestines (duodenum, jejunum, ileum, and colon), especially so in the duodenum (**Fig. 3A**). Treatment with *P. acnes* alone induced IL-18 more than twofold in each organ except for the lung and colon. Treatment with *P. acnes* and LPS induced a marked increase in IL-18 levels in the liver and spleen but decreased them slightly in the duodenum, jejunum, and ileum, compared with those with *P. acnes* alone.

Western blot analysis showed that proIL-18 was expressed at a low amount in the livers of untreated mice, and *P. acnes* treatment induced proIL-18 and mature IL-18 in the liver (Fig. 3B). Treatment with *P. acnes* and LPS resulted in a further increase of both forms of IL-18. The spleens of untreated mice expressed proIL-18 and a band lower than 18 kDa, suggesting that mature IL-18 is not expressed in the untreated spleen. The expression of proIL-18 and mature IL-18 was induced by *P. acnes* alone and increased further by *P. acnes* and LPS in the spleen. In contrast, both forms existed already in the untreated duodenum, and mature IL-18 was increased markedly in the duodenum by *P. acnes* alone and by *P. acnes* and LPS. In the jejunum and ileum, mature IL-18 was mainly expressed, and the expression was increased and decreased slightly by *P. acnes* alone and *P. acnes* and LPS, respectively, compared with the untreated control. In the colon, the expression of pro-IL-18 was higher than that of mature IL-18 in the untreated control, and *P. acnes* treatment reduced pro-IL-18 and increased mature IL-18 expressions. The expression of proIL-18 was increased by *P. acnes* and LPS, compared with *P. acnes* alone. These results indicate that *P. acnes* priming in mice leads to the accumulation of mature IL-18 in these organs and renders the mice susceptible to LPS, that LPS challenge induced the release of mature IL-18, and that the diverse expression of pro- and mature IL-18 occurs in each organ and even in the intestines.

Effect of macrophage depletion on the IL-18 levels in organs in mice

As Figure 2 illustrates that Clo-lip showed no effect on serum IL-18 levels, the effect of Clo-lip on the IL-18 levels in various organs in mice was then examined. Treatment of mice with *P. acnes* and LPS markedly increased the IL-18 levels in the liver and spleen but not in the lung, and the administration of Clo-lip, three times (1 day before and 2 and 4 days after *P.*

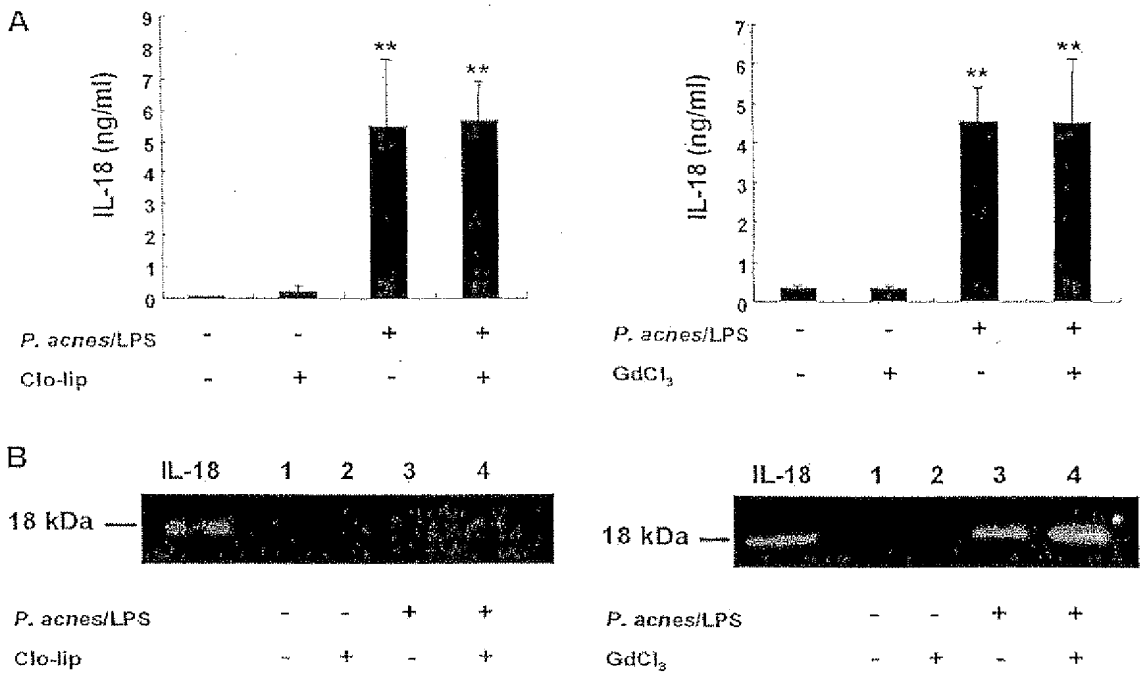

Fig. 2. Effect of Clo-lip and $GdCl_3$ on the induction of serum IL-18 in mice. (A) *P. acnes* (1 mg dry weight/mouse) or PBS was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v. with LPS (1 μg/mouse) or PBS. Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administerted i.p. into the mice 1 day before and 2 and 4 days after *P. acnes* administration. $GdCl_3$ (10 mg/kg) was administerted i.v. into the mice 2 days before and 2 and 5 days after *P. acnes* administration. Blood was then taken from the mice 2 h after LPS challenge, and the levels of IL-18 in the sera were determined by ELISA. The results were expressed as the means ± SD for five mice. **, $P < 0.01$, compared with untreated mice. (B) Samples in A were subjected to Western blotting using anti-mouse IL-18 pAb. mrIL-18 (1 ng) was loaded as a control. The results are representative of five mice of each group.

acnes injection) in the mice, slightly, but not significantly, decreased in the IL-18 levels in the liver (Fig. 4A). However, a substantial amount of IL-18 remained in the liver following the Clo-lip treatment. In addition, the IL-18 levels in the spleens of mice treated with *P. acnes* and LPS did not change following the Clo-lip treatment. The IL-18 levels in the intestines are also unchanged by the Clo-lip treatment (data not shown). Consistent with this, Western blot analysis showed that the expression of mature IL-18 was almost unchanged in the livers and spleen of mice treated with *P. acnes* and LPS after the Clo-lip administration (Fig. 4B). These results further indicate that IL-18 expressed in activated macrophages in organs and tissues does not cause the elevation of serum IL-18 levels.

Effect of macrophage depletion on LPS-induced liver injury in *P. acnes*-primed mice

As it has been reported that treatment with *P. acnes* and LPS induces IL-18-dependent, acute liver injury in mice through the induction of hepatotoxic factors such as TNF-α and that Kupffer cells are the major source of IL-18 in the liver [28–30], we next examined the effect of Clo-lip on the liver injury. The levels of the liver enzymes, AST and ALT, in sera were increased markedly by treatment with *P. acnes* and LPS, and their levels were decreased significantly by the three-time administration of Clo-lip (Fig. 5A). However, the serum AST and ALT levels were still high compared with those of untreated mice. Treatment with *P. acnes* and LPS resulted in a marked increase in serum TNF-α levels, and the induction of serum TNF-α was slightly, but not significantly, reduced by Clo-lip treatment (Fig. 5B). Consistent with this, histological analysis showed that treatment with *P. acnes* and LPS induced severe liver injury (necrotic change and granuloma formation) and cell infiltration, and treatment with *P. acnes* alone also induced granuloma formation and cell infiltration (Fig. 6). No granuloma was observed, and the cell infiltration was reduced by Clo-lip administration. However, the cell infiltration and necrotic change were still observed in the Clo-lip-administered liver. These results suggest that Kupffer cells are actually involved in liver injury in mice treated with *P. acnes* and LPS and that IL-18 and TNF-α from non-Kupffer cells also contribute to the liver injury.

DISCUSSION

IL-18 was identified originally as a potent IFN-γ-inducing factor in the serum and livers of mice, which had been administered *P. acnes* and LPS sequentially [1]. IL-18 was first identified in activated macrophages, such as Kupffer cells, in the liver. Further investigations have revealed that IL-18 is also expressed in nonimmune cells, such as keratinocytes, osteoblasts, adrenal cortex cells, epithelial cells of various organs and tissues, microglial cells, and synovial fibroblasts [2–4]. Increased levels of IL-18 have been reported in the sera

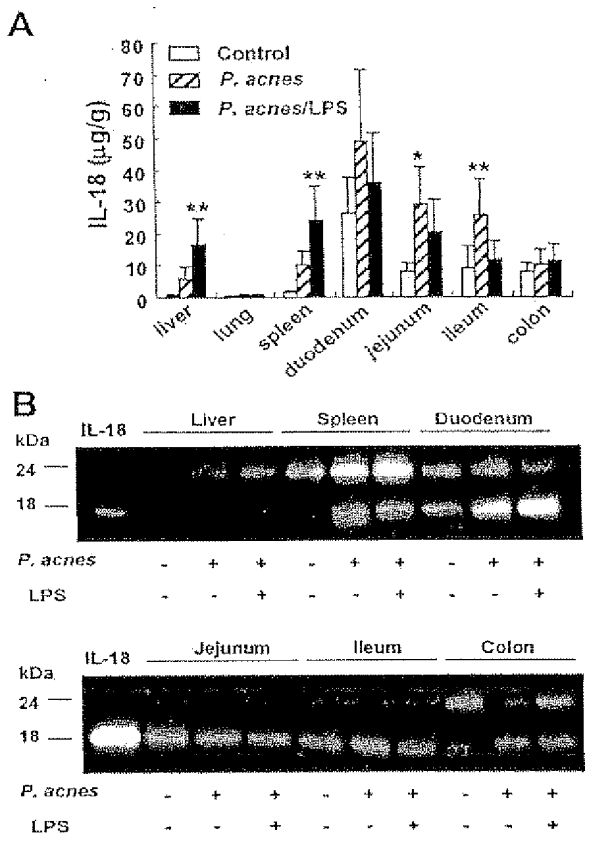

Fig. 3. IL-18 and levels in various organs or tissues in mice. (A) *P. acnes* (1 mg dry weight/mouse) or PBS was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v. with LPS (1 μg/mouse) or PBS. Organs or tissues were then taken from the mice 2 h after LPS challenge, and the levels of IL-18 in the samples were determined by ELISA. The results were expressed as the means ± SD for five mice. *, $P < 0.05$, and **, $P < 0.01$, compared with untreated mice. (B) Samples in A were subjected to Western blotting using anti-mouse IL-18 pAb. mrIL-18 (10 ng) was loaded as a control. The results are representative of five mice of each group.

from patients with a wide variety of diseases, including autoimmune and inflammatory disorders [6–11], allergy [12], allograft rejection [13], and infectious diseases [14–16], and the elevated serum IL-18 levels are considered to be a parameter for the disease severity and a diagnostic marker. However, it is unclear whether the major source of serum IL-18 is immune cells, such as activated macrophages, or nonimmune cells, such as epithelial origin. The present study used Clo-lip to deplete phagocytic macrophages selectively in mice. After the ingestion of Clo-lip into macrophages, phospholipases in the lysosomes degrade the phospholipid bilayers, releasing the clodronate into the cells, resulting in cell death, and the i.v. injection of Clo-lip into mice results in a selective depletion of macrophages, including liver and spleen macrophages [21, 22]. This study demonstrated that the three-time administration of Clo-lip eliminated F4/80$^+$ macrophages in the liver and spleen (Fig. 1), suggesting that the Clo-lip treatment depletes phagocytic macrophages in mice and that phagocytic macrophages are not critically involved in the increase in serum IL-18 levels. The present study also used $GdCl_3$ to inactivate phagocytic macrophages, as it is reported that the i.v. injection of $GdCl_3$ not only blocks phagocytosis but also eliminates these cells [24, 25]; however, we were unable to deplete F4/80$^+$ macrophages in the liver and spleen by the i.v. injection of $GdCl_3$ three times (Fig. 1), and the administration of $GdCl_3$ three times showed no obvious effect on serum IL-18 levels, which were comparable with Clo-lip (Fig. 2). It is also reported that $GdCl_3$ does not significantly reduce the number of phagocytically active cells in the liver [31], that splenic macrophages are less vulnerable to $GdCl_3$ [25], and that $GdCl_3$ treatment results in a significant increase in serum levels of TNF-α, IL-6, and liver enzymes, ALT and AST [32]. Therefore, we consider that Clo-lip is more effective in the inactivation and elimination of phagocytic macrophages than $GdCl_3$, and we used Clo-lip mainly.

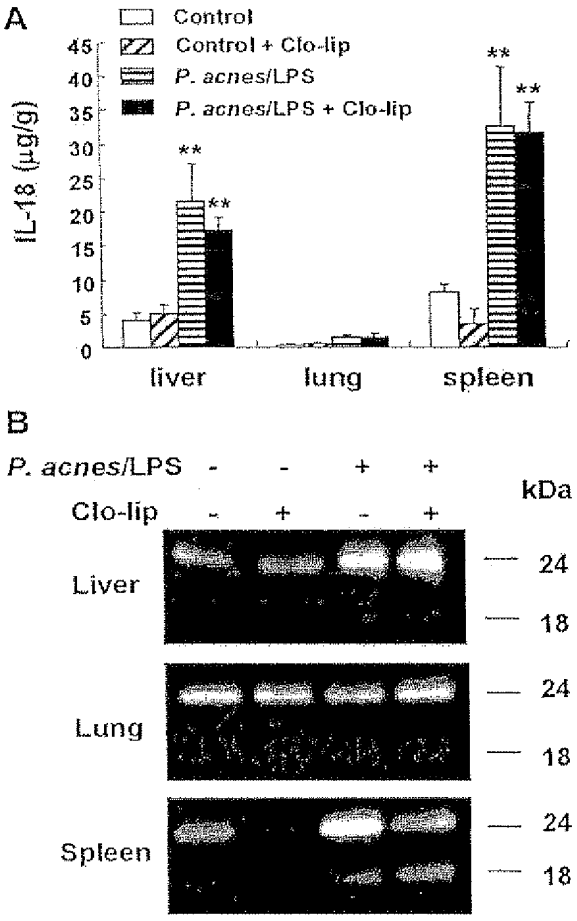

Fig. 4. Effect of macrophage depletion on the IL-18 levels in the liver, lung, and spleen in mice. (A) *P. acnes* (1 mg dry weight/mouse) or PBS was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v. with LPS (1 μg/mouse) or PBS. Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administered i.p. to the mice 1 day before and 2 and 4 days after *P. acnes* administration. The liver, lung, and spleen were then taken from the mice 2 h after LPS challenge, and the levels of IL-18 in the samples were determined by ELISA. The results are expressed as the means ± SD for five mice. **, $P < 0.01$, compared with untreated (Control) mice. (B) Samples in A were subjected to Western blotting using anti-mouse IL-18 pAb. The results are representative of five mice of each group.

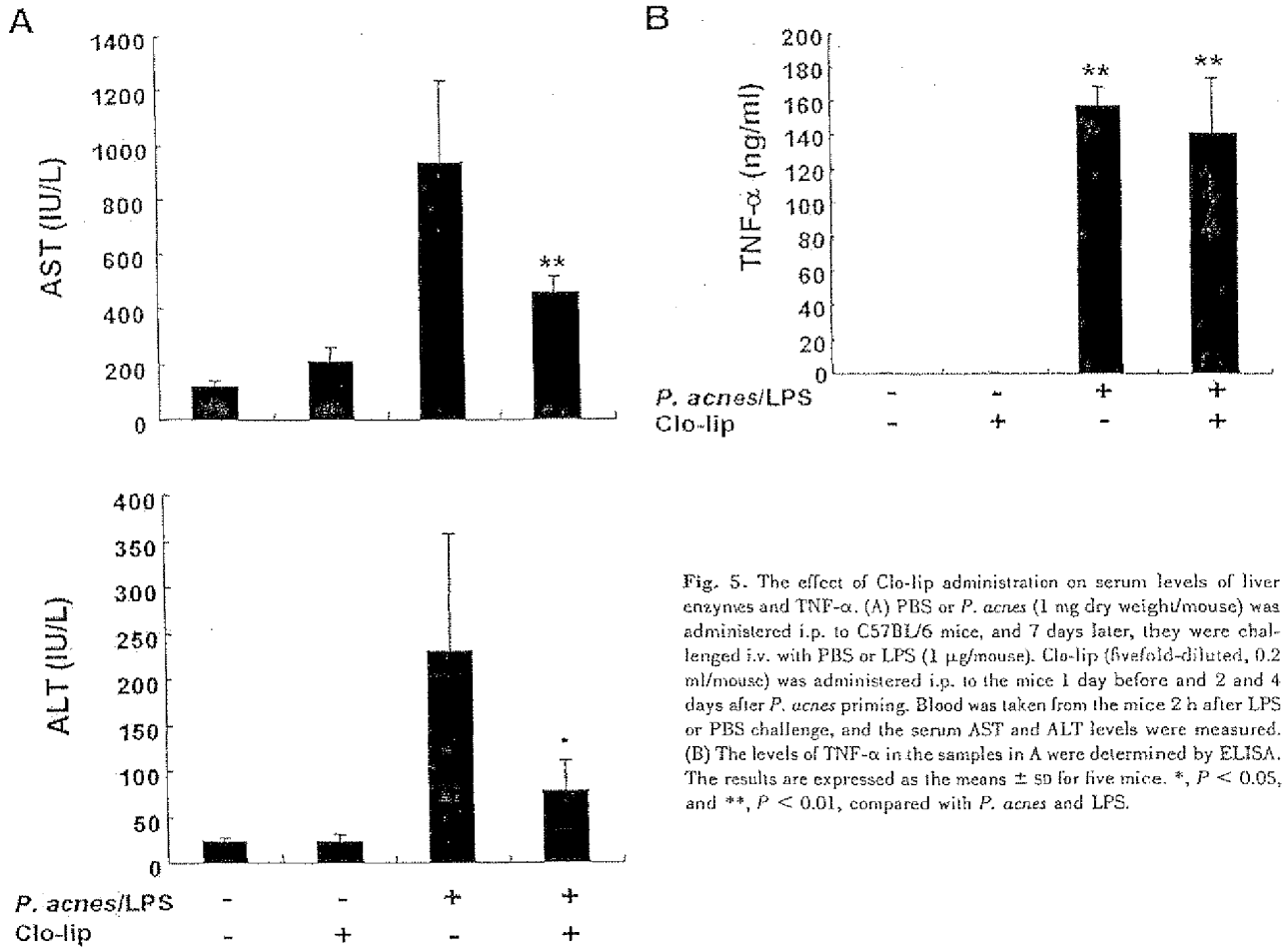

Fig. 5. The effect of Clo-lip administration on serum levels of liver enzymes and TNF-α. (A) PBS or *P. acnes* (1 mg dry weight/mouse) was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v. with PBS or LPS (1 μg/mouse). Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administered i.p. to the mice 1 day before and 2 and 4 days after *P. acnes* priming. Blood was taken from the mice 2 h after LPS or PBS challenge, and the serum AST and ALT levels were measured. (B) The levels of TNF-α in the samples in A were determined by ELISA. The results are expressed as the means ± SD for five mice. *, $P < 0.05$, and **, $P < 0.01$, compared with *P. acnes* and LPS.

Analysis using IFN-γ-deficient mice showed that the induction of LPS hypersensitivity by *P. acnes* priming is mediated strictly by IFN-γ [33, 34]. IL-18 with IL-12 strongly induces IFN-γ from activated $CD4^+$ Th1 cells and NK cells [1], and IFN-γ also in turn can regulate the secretion of bioactive IL-18, constituting a feedback loop between these cytokines [33]. IFN-γ plays a major role in immune regulation. It activates various macrophage functions, including cytokine production, antimicrobial activity, and antigen processing and presentation [35]. In addition, IFN-γ activates nonimmune cells, including keratinocytes, epithelial cells, and fibroblasts [36–38]. IFN-γ-activated keratinocytes express a number of chemokines, cytokines, and adhesion molecules [36, 37]. The priming of oral epithelial cells and gingival fibroblasts with IFN-γ induces sensitivity to pathogen-associated molecular patterns, including LPS and peptidoglycans [38, 39]. Therefore, the induction of IFN-γ during *P. acnes* priming in mice is critically involved in the accumulation of a mature IL-18 from each organ.

The IL-18 levels were increased in the liver and spleen but decreased in the intestines by *P. acnes* and LPS compared with *P. acnes* alone (Fig. 3A). Immunoblot analysis showed that proIL-18 was detected mainly in the liver and spleen in untreated mice (Figs. 3B and 4B), whereas the diverse expression of proIL-18 and mature IL-18 was observed in the intestines of mice (Fig. 3B). Furthermore, treatment with *P. acnes* and LPS induced a marked increase in IL-18 levels in the liver and spleen but decreased them slightly in the intestines compared with those with *P. acnes* alone. Therefore, it is possible that the mechanism of accumulation and release of IL-18 are different depending on the organ and even in the intestines.

F4/80 is expressed on most resident tissue macrophages, including the red pulp macrophages in the spleen, Kupffer cells in the liver, and Langerhans cells in the skin [40]. This study showed that the Clo-lip treatment did not reduce the IL-18 levels in the livers and spleens of mice treated with *P. acnes* and LPS (Fig. 4). These findings suggest that the tissues or cells other than activated macrophages also express IL-18 in the liver and spleen. DC are able to produce IL-18 [41] and are not phagocytic [42], and the phenotypes of hepatic DC are $F4/80^{low}$ or $F4/80^-$ [43]. A recent study revealed that DC in the liver play important roles in the induction and regulation of immune responses [43]. Therefore, it is possible that the administration of Clo-lip did not efficiently eliminate the IL-18-expressing DC in the liver. This possibility may be the case in the spleen, as there is no report that T and B cells produce IL-18. However, murine bone marrow-derived DC produce IL-18 at ~100 pg/ml levels for 7 or 8 days of culture [41], whereas serum IL-18 levels in mice treated with *P. acnes* and

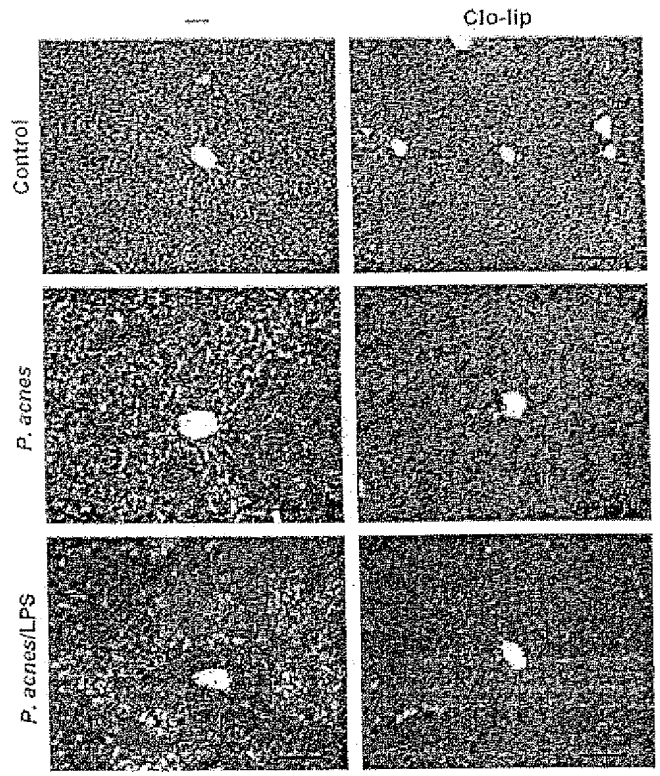

Fig. 6. Effect of Clo-lip on LPS-induced liver injury in *P. acnes*-primed mice. PBS or *P. acnes* (1 mg dry weight/mouse) was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v., with PBS or LPS (1 μg/mouse). Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administered i.p. to the mice 1 day before and 2 and 4 days after *P. acnes* priming. Liver specimens were then sampled 2 h after PBS or LPS challenge, and liver tissue sections were stained with H&E. The results are representative of five mice of each group. Original scale bars, 200 μm.

LPS were ng/ml levels (Fig. 2). Therefore, it is conceivable that the contribution of CD-derived IL-18 to serum IL-18 levels is marginal. Another possible source in the liver is oval cells, which are proliferating, epithelial cells with an ovoid nucleus and appear in liver generation, and IL-18 was expressed in oval cells in the regenerating liver at mRNA and protein levels [44]. Therefore, it is also possible that pathological changes caused by *P. acnes* induce IL-18-expressing oval cells in the liver. NK cells or NK T cells may also be the source of IL-18. Further studies are required to clarify these points.

It has been reported that treatment with *P. acnes* and LPS induces IL-18-dependent liver injury through the induction of TNF-α [28–30] and suggested that Fas/Fas ligand-mediated IL-18 secretion from Kupffer cells causes the liver injury in mice [29]. Analysis using IL-18 transgenic mice showed that IL-18 plays a key role in regulating hepatocyte apoptosis in vivo [45], indicating that IL-18 is critically involved in the liver injury. This study showed that depletion of $F4/80^+$ macrophages by Clo-lip in *P. acnes* and LPS-treated mice reduced serum AST, ALT, and TNF-α levels and pathological change of the liver (Figs. 5 and 6). These results suggest that Kupffer cells play an important role for the onset of liver injury. However, a substantial amount of the liver enzymes and the liver injury was found in the Clo-lip-treated mice, suggesting that IL-18 and TNF-α from liver tissues or cells other than Kupffer cells also cause the liver injury. It is also possible that IL-18 in circulation is involved in the liver injury, as the i.p. injection of rIL-18 is able to induce the liver injury in *P. acnes*-primed mice [29].

In conclusion, the present study suggests that serum IL-18 is derived from nonphagocytic macrophages, probably from epithelial cells of various organs and tissues in mice treated with *P. acnes* and LPS. Increased levels of serum IL-18 are associated with a wide variety of diseases [6–16]; therefore, it is possible that the serum IL-18 is derived from diseased organs or tissues. IL-18 is not only an important regulator of innate and acquired immune responses but also a potent, proinflammatory cytokine, which regulates a wide variety of autoimmune and inflammatory diseases [2–4]. Bone malformation and the exacerbation of colitis were reported in IL-18 transgenic mice [46, 47], and the overexpression of IL-18 with the Keratin 5 promoter in mice showed exacerbated and prolonged, allergic and nonallergic, inflammatory skin reactions [48]. These findings indicate that the overexpression of IL-18 results in deleterious alterations in the organs and tissues. Therefore, IL-18 overexpressed in these organs and tissues may be an important, therapeutic target for the treatment of diseases.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (17390483, 18591995, and 18791379) and the 21st Century Centers of Excellence Program Special Research grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank T. Hoshino for providing us with anti-mouse IL-18 polyclonal antibody.

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Reference 1